Immunization With Melan-A Peptide-Pulsed Peripheral Blood Mononuclear Cells Plus Recombinant Human Interleukin-12 Induces Clinical Activity and T-Cell Responses in Advanced Melanoma

By Amy C. Peterson, Helena Harlin, and Thomas F. Gajewski

Purpose: Preclinical studies showed that immunization with peripheral blood mononuclear cells (PBMC) loaded with tumor antigen peptides plus interleukin-12 (IL-12) induced CD8+ T-cell responses and tumor rejection. We recently determined that recombinant human (rh) IL-12 at 30 to 100 ng/kg is effective as a vaccine adjuvant in patients. A phase II study of immunization with Melan-A peptide-pulsed PBMC + rhIL-12 was conducted in 20 patients with advanced melanoma.

Patients and Methods: Patients were HLA-A2–positive and had documented Melan-A expression. Immunization was performed every 3 weeks with clinical re-evaluation every three cycles. Immune responses were measured by ELISpot assay before and after treatment and through the first three cycles, and were correlated with clinical outcome.

Results: Most patients had received prior therapy and had visceral metastases. Nonetheless, two patients achieved a complete response, five patients achieved a minor or mixed response, and four patients had stable disease. The median survival was 12.25 months for all patients and was not yet reached for those with a normal lactate dehydrogenase. There were no grade 3 or 4 toxicities. Measurement of specific CD8+ T-cell responses by direct ex vivo ELISpot revealed a significant increase in interferon gamma–producing T cells against Melan-A ($P = .015$) after vaccination, but not against an Epstein-Barr virus control peptide ($P = .86$). There was a correlation between the magnitude of the increase in Melan-A–specific cells and clinical response ($P = .046$).

Conclusion: This immunization approach may be more straightforward than dendritic cell strategies and seems to have clinical activity that can be correlated to a biologic end point.

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MOST MELANOMA tumors express antigens that can be recognized by CD8+ T cells.[1,2] Nonetheless, tumors frequently escape immune destruction, either from a failure to generate an optimal tumor antigen-specific T-cell response or from development of resistance to the T-cell response induced. One strategy to overcome the former hurdle is through active immunization, the opportunity for which has been facilitated by the molecular definition of melanoma antigens.[3] Specific CD8+ T cells that are properly activated can home to tumor sites and kill tumor cells, to the extent to which they can overcome negative immunoregulatory pathways and tumor resistance.[4]

The optimal immunization strategy for inducing tumor antigen-specific CD8+ effector T cells in humans remains undefined. However, antigen-presenting cell-based strategies have shown promise. Both monocyte-derived[5,6] and bone marrow–derived[7] dendritic cells (DCs) have been loaded with melanoma tumor antigens and administered in the advanced-disease setting, with evidence for immunization and tumor regression in subsets of patients. However, DCs are cumbersome to generate and alternative approaches that are more straightforward yet equally as effective would be useful. One cofactor produced by DCs that contributes to their efficacy is interleukin-12 (IL-12), which facilitates the induction of interferon gamma (IFN-γ)–producing cytolytic effector cells.[8-10] Endogenous IL-12 seems necessary for optimal rejection of immunogenic murine tumors[11,12] and provision of exogenous IL-12, either alone[13] or combined with tumor antigen-based vaccines,[14-19] can induce rejection of pre-established tumors in murine models. We previously have shown that coadministration of IL-12 with peripheral blood mononuclear cells (PBMCs) loaded with tumor antigen peptides induced specific cytolytic T-lymphocyte responses and tumor protection in mice, circumventing the need to generate dendritic cells.[20] The ease by which PBMC can be isolated from patients has made this an attractive approach for clinical translation. We recently conducted a phase I clinical study to determine the dose of recombinant human (rh) IL-12 necessary to induce T-cell responses in combination with antigen-loaded PBMCs, and found that doses from 30 to 100 ng/kg administered subcutaneously (sc) at the vaccine site were optimal and well tolerated.[21] The effective range of doses indicated that a straight dose of 4 μg might be used.

In this article, we describe results of a phase II clinical study of immunization with Melan-A/MART-1[3] peptide-pulsed autologous PBMCs + rhIL-12 in HLA-A2–positive patients with

From the University of Chicago, Departments of Pathology and Medicine, Section of Hematology/Oncology, and the Ben May Institute for Cancer Research, Chicago, IL.

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A.P. and H.H. contributed equally to this work.

Address reprint requests to Thomas F. Gajewski, MD, PhD, University of Chicago, 5841 S. Maryland Ave., MC2115, Chicago, IL 60637; email: tgajewsk@medicine.bsd.uchicago.edu.

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advanced melanoma. Immune responses were analyzed using a direct ex vivo ELISpot assay. We show that this vaccine approach had clinical activity and that the magnitude of increased T-cell response correlated with clinical outcome.

PATIENTS AND METHODS

Patient Enrollment and Eligibility

This was an open-label, nonrandomized, single-institution study of Melan-A peptide-pulsed autologous PBMCs + rhIL-12.[4] The protocol was approved by the University of Chicago Institutional Review Board and all patients signed written informed consent. Patients who were both HLA-A2–positive and showed Melan-A tumor expression by reverse transcriptase polymerase chain reaction (RT-PCR) were considered for inclusion. Additional inclusion criteria were life expectancy more than 12 weeks, Karnofsky performance status ≥70, and adequate hematopoietic, renal, and hepatic function. Delayed-type hypersensitivity (DTH) skin testing was performed against mumps, *Candida*, and *Trichophytin*, not for eligibility but to correlate subsequently with clinical outcome and immunization potential. Patients were excluded if they had severe cardiovascular disease or arrhythmia, were pregnant or nursing, had biologic therapy received within 4 weeks, tested positive for hepatitis B surface antigen or human immunodeficiency virus (HIV), had clinically significant autoimmune disease or any illness requiring immunosuppressive therapy, had a psychiatric illness that would interfere with patient compliance and informed consent, had active gastrointestinal bleeding or uncontrolled peptic ulcer disease, or had uncontrolled brain metastases. Patients with treated brain metastases who were clinically and radiographically stable and did not require corticosteroids were allowed to enter onto the trial.

Patient Characteristics

Twenty patients with metastatic melanoma were enrolled after giving written informed consent. Patient characteristics are outlined in Table 1. All patients had advanced disease; the majority had at least three sites of metastasis, 60% of which were visceral (ie, noncutaneous and nonpulmonary metastases). Approximately two thirds of the patients had received prior therapy, and 10 patients had an elevated lactate dehydrogenase (LDH) level, which is an important negative prognostic factor.[22] Only 45% were positive for at least one recall antigen (mumps, *Candida*, or *Trichophytin*) by DTH skin testing.

Table 1. Patient Characteristics

Patient Characteristic	Patients (n = 20) No.	%
Age, years		
Median	58	
Range	35-79	
Sex		
Male	9	45
Female	11	55
Karnofsky performance status (ECOG)		
90%-100% (0)	10	50
70%-80% (1)	9	45
60%-70% (2)	1	5
No. of metastatic sites		
1	2	10
2	None	
≥3	18	90
Location of metastases		
Visceral	13	65
Brain (treated)	4	20
Prior therapy		
None	6	30
Chemotherapy or immunotherapy	7	35
As only prior therapy	5	25
Chemotherapy	1	5
As only prior therapy	1	5
Immunotherapy	4	20
As only prior therapy	1	5
Other*	2	10
As only prior therapy	None	
Adjuvant IFN-α	5	25
As only prior therapy	3	15
Elevated LDH	10	50
DTH recall positive	9	45

Abbreviations: ECOG, Eastern Cooperative Oncology Group; IFN-α, interferon alfa-2b; LDH, lactate dehydrogenase; DTH, delayed-type hypersensitivity.

*Experimental therapy other than a melanoma vaccine, immunomodulatory cytokine, or chemotherapy.

RT-PCR Analysis

RNA was isolated from fresh tumor cells using guanidine and cesium chloride. cDNA was synthesized and PCR was performed for Melan-A and beta-actin using the primer pairs and reaction conditions described previously.[21] Control reactions without reverse transcriptase were performed to rule out a contribution of genomic DNA. PCR products were visualized using a 1.5% ethidium bromide–stained agarose gel. No formal quantitation was performed.

Vaccine Preparation

Therapy consisted initially of three 21-day cycles. Vaccinations were given on the first day of each cycle and rhIL-12 was administered subcutaneously on days 1, 3, and 5. Approximately 100 to 150 mL of peripheral blood from patients was collected on day 1 of each cycle into heparinized 60-mL syringes using sterile technique. PBMCs were isolated over a Lymphoprep gradient (Lymphoprep; Axis-Shield PoC, Oslo, Norway), counted, washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) at 40×10^6 cells/mL. At least 10×10^6 cells from each sample were cryopreserved to prepare $CD8^+$ and $CD8^-$ fractions for subsequent correlative immunologic studies. The Melan-A_{27-35} peptide (AAGIGILTV) was produced according to good manufacturing practice standards by Multiple Peptide Systems (San Diego, CA) and provided in lyophilized vials. Aliquots of peptide were prepared at 5 mmol/L in dimethyl sulfoxide and stored at −80°C for up to 3 months. Peptide preparations were quality controlled for HLA-A2 binding, sterility, and identity by high-performance liquid chromatography and mass spectrometry. An aliquot of peptide was diluted to 20 μmol/L in DPBS and mixed with an equal volume of patient PBMCs (final peptide concentration, 10 μmol/L; target number of PBMCs, 10^8) followed by incubation at 37°C for 1 hour in 10 mL DPBS. The cells were then irradiated (20 Gy), washed in DPBS, and resuspended in 1 mL DPBS. The suspension of peptide-loaded PBMCs was injected sc using a 1-mL syringe and a 21-gauge needle, divided evenly into two sites. Preferred sites were those near draining lymph node basins but not near a tumor mass. The actual number of PBMCs administered per vaccine ranged from 78 to 100×10^6.

rhIL-12 was provided by Genetics Institute (Cambridge, MA) as a lyophilized powder of 10 μg under vacuum. Each vial was intended for single use only and was stored as a powder in our research pharmacy at 2 to 8°C until reconstituted with sterile water for injection. Once reconstituted, rhIL-12 was loaded into 3-mL syringes and used within 4 hours. rhIL-12 (4 μg) was administered sc with a 25-gauge needle just after pulsed PBMC inoculation and immediately adjacent to one of the two immunization sites on days 1, 3, and 5. The same approximate location was used for each injection of peptide-pulsed PBMCs and rhIL-12 for each cycle.

Toxicity Assessment and Criteria for Clinical Response

Toxicities were determined using the National Cancer Institute common toxicity criteria scale version 2.0. A complete response (CR) was assigned if there was disappearance of all lesions without the appearance of any new

lesions; a partial response (PR) was defined as ≥ 50% reduction in total tumor volume; a minor response (MR) was defined as less than 50% reduction in total tumor volume; progressive disease (PD) was assigned if new lesions appeared, any tumor reappeared, or if a 25% increase in tumor area was observed; a mixed response was assigned if at least one tumor decreased in size with other or new tumors growing; stable disease (SD) was anything that did not fit the aforementioned criteria. When possible, cutaneous lesions were photographed.

$CD8^+$ T-Cell Preparation

$CD8^+$ and $CD8^-$ fractions from PBMC were isolated at the time of preparation of each vaccine and cryopreserved until analysis in batch fashion. $CD8^+$ T lymphocytes were isolated by positive selection using CD8 microbeads and magnetic columns (MACS system; Miltenyi Biotech, Auburn, CA). The unbound $CD8^-$ fraction was cryopreserved for use as antigen-presenting cells for in vitro expansion of specific $CD8^+$ T cells. Although the primary ELISpot analysis was performed directly with thawed cells, a secondary assay was carried out after in vitro expansion. For in vitro expansion, $CD8^-$ cells were thawed from each time point and pooled, pulsed with 50 μmol/L Melan-A peptide in serum-free Iscove's modified Dulbecco's medium (IMDM) with $beta_2$-microglobulin, irradiated (3,000 rad), washed, and plated at 2×10^6 cells/well in 24-well plates. $CD8^+$ T cells were thawed and cultured with the irradiated $CD8^-$ cells at 4×10^5 cells/well in IMDM medium containing 10% human AB serum. After 5 days, the cells were collected and plated with a new batch of Melan-A–pulsed irradiated $CD8^-$ cells. After an additional 5 days the cells were collected and tested.

ELISpot Assays

Briefly, 96-well membrane bottomed plates (MAHA S4510; Millipore, Bedford, MA) were coated with 15 μg/mL of antihuman IFN-γ antibody (Mabtech, Cincinnati, OH) in PBS. The plates were washed and $CD8^+$ T cells, either freshly thawed at 5×10^4 cells/well or after in vitro expansion at 5×10^3 cells/well, were plated in triplicate in IMDM medium with 10% human AB serum. T2 cells (transporter associated with antigen processing–deficient cell line, American Type Culture Collection no. CRI 1992) were pulsed for 1 hour at 37°C with 50 μmol/L peptide (either derived from HIV [ILKEPVHGV], Epstein-Barr virus [EBV; GLCTLVAML], or Melan-A [AAGIGILTV]), washed, and plated at a 5-to-1 ratio to the T cells. A replicate of $CD8^+$ T cells was stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) + ionomycin (0.5 μg/mL) as a positive control. After 24 hours, the cells were removed by washing with PBS + 0.05% Tween (wash buffer), and biotinylated antihuman IFN-γ antibody was added in PBS + 0.5% fetal calf serum. The plates were incubated for 2 to 4 hours at room temperature, washed, and streptavidin–alkaline phosphatase was added for 1 hour at room temperature. The plates were then washed, BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium) was added, and the plates were finally washed with water and allowed to air dry. Plates were scanned with an ELISpot reader (CTL Technologies, Cleveland, OH) and the number of spots per well was enumerated after the background was set on the basis of wells that had been incubated with medium alone; spot separation was adjusted using Immunospot software (CTL Technologies). For each sample, the number of T cells producing IFN-γ in response to EBV or Melan-A peptides was determined by subtracting the number of spots seen in response to HIV peptide. The mean and SD were determined for each triplicate sample. After immunization, the time point at which peak frequencies among the first three cycles were observed was used for data analysis.

Statistical Analysis

Comparisons between pre- and post-ELISpot frequencies were performed using a paired *t* test, and comparisons of augmented ELISpot frequencies between responders and nonresponders were made using an unpaired two-sided *t* test. Correlations between various dichotomous variables and clinical outcome were made using Fisher's exact test (two-sided). Survival data were determined using the Kaplan-Meier method, with differences among subgroups assessed by the log-rank test. All analyses were performed using SPSS software (version 8.0; SPSS Inc, Chicago, IL).

Table 2. Adverse Events

Adverse Event	Grade 1	Grade 2	Grade 3
Fatigue	16	0	0
Anorexia	6	0	0
Fever	7	0	0
Rash	3	0	0
Headache	3	0	0
Nausea	2	0	0
Injection site reaction	5	0	0
Neutropenia	1	2	0
Thrombocytopenia	2	0	0
Hepatic	5	2	0
Creatinine	1	0	0

NOTE. Adverse events were determined using the National Cancer Institute common toxicity criteria scale version 2.0.

RESULTS

Immunization Treatment and Toxicities

Each 3-week cycle consisted of immunization on day 1 and sc rhIL-12 administration on days 1, 3, and 5, as described in Methods. Three cycles constituted one course of therapy and patients were evaluated for response after each course. Patients were observed as inpatients in our General Clinical Research Center for the first 24 hours of each cycle.

Adverse reactions are listed in Table 2. All but one patient completed at least three cycles of therapy. There were no grade 3 to 4 toxicities; two patients had grade 2 neutropenia and two patients had grade 2 ALT or AST elevations, which were reversible. The most common adverse reactions were fatigue and fever.

Clinical Outcome

Clinical response outcomes are listed in Table 3. Two patients had a CR, for an overall response rate of 10%. In addition, four patients (20%) had a mixed response, one patient (5%) had an MR, four patients (20%) had SD, and the remaining nine patients (45%) had PD. The sites of tumor response were diverse. The two patients who experienced a CR both had numerous metastases of 2 cm or less and a normal LDH. One patient was female, had multiple cutaneous lesions, and no prior therapy; the other patient was male, had multiple lung lesions, and had experienced prior treatment failure from chemoimmunotherapy. Neither patient experienced a recurrence with a mean follow-up time of 28 months at the time of data analysis. Of the five other patients who showed a decrease in size of at least one tumor mass, three had responses in skin, one had a response in bone, and one had a response in an adrenal lesion. Three of the four patients with SD had visceral metastases.

Table 3. Clinical Outcome

Best Response	No. of Patients	%
Complete response	2	10
Partial response	0	0
Minor response	1	5
Mixed response	4	20
Stable disease	4	20
Progressive disease	9	45

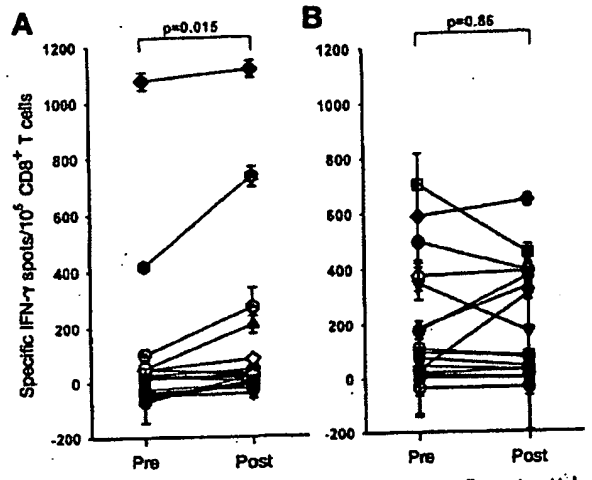

Fig 1. Interferon gamma ELISpot frequencies by $CD8^+$ T cells against Melan-A and (A) Epstein-Barr virus (EBV) (B) pre- and postimmunization. Control values with HIV peptide were subtracted out. Post- and pretreatment values were compared using a paired *t* test.

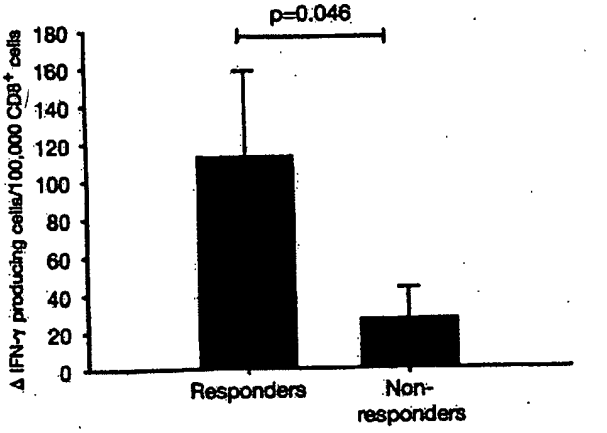

Fig 2. Comparison of increased Melan-A ELISpot frequencies after immunization between clinical responders and nonresponders. The absolute difference between Melan-A-specific ELISpot frequencies post- and pretreatment was compared between responders and nonresponders using a two-sided, unpaired *t* test.

Peptide-Specific T-Cell Responses by ELISpot

A carefully controlled IFN-γ ELISpot assay was used to monitor the immune response to immunization. Cryopreserved $CD8^+$ T cells were thawed in batch fashion and stimulated in triplicate directly ex vivo with T2 cells loaded with peptides derived from either HIV, EBV, or Melan-A. The HIV values were subtracted from those obtained with either Melan-A or EBV as an internal control at each time point. Seventeen of the enrolled patients had adequate cryopreserved material with which to perform immunologic assessments.

As shown in Fig 1, some patients displayed a high frequency of Melan-A–specific $CD8^+$ T cells before vaccination, with as high as 1% of $CD8^+$ cells responding to this peptide. These T cells were functional because they produced IFN-γ. The majority of patients showed an increase in the frequency of Melan-A–specific cells after immunization ($P = .015$). In contrast, the frequencies of specific $CD8^+$ T cells responding to the EBV peptide did not vary significantly overall ($P = .86$). Although the changes in T-cell frequency were modest, these results demonstrate an antigen-specific response after immunization with Melan-A peptide-pulsed PBMC + rhIL-12.

The changes in Melan-A–specific ELISpot frequencies were compared among patients who had a mixed response or better and those who had no clinical response. As shown in Fig 2, the mean increase in Melan-A–specific T cells for the clinical responders was 112 ± 45 and for nonresponders was 26 ± 16, indicating that a greater absolute increase in Melan-A–specific T cells was associated with tumor regression ($P = .046$).

Survival and Associations Between Immunologic Parameters and Clinical Outcome

The overall median survival was 12.25 months and is shown in Fig 3A. Seven patients remained alive at the time of data analysis, with all patients followed beyond 12 months. Because the presence of elevated levels of serum LDH is a known negative prognostic factor,[23] survival was also compared in response to this vaccine on the basis of LDH level (Fig 3B). The median survival for patients with an elevated LDH level was 9.25 months, whereas the median had not yet been reached for those with a normal LDH ($P = .005$). In addition, the median survival for patients who experienced a significant increase in Melan-A–specific T cells was not yet reached, compared with 8.5 months for patients without a significant increase in Melan-A–specific cells (Fig 3C; $P = .120$).

Additional immunologic parameters that had been measured were also analyzed for associations with either clinical response or survival and are summarized in Table 4. Neither a positive recall DTH to standard antigens nor a relatively high number of EBV- or Melan-A–specific $CD8^+$ T cells before immunization correlated with either outcome. The median pretreatment Melan-A–specific T cell frequency was 23 in clinical nonresponders and −26 in responders. To increase the sensitivity of the assay to detect Melan-A–specific T cells, an in vitro expansion was performed on the preimmunization samples and analyzed by ELISpot as described in Methods. Ten patients showed high Melan-A–specific T cell frequencies after in vitro expansion. However, this also failed to correlate with clinical outcome. Finally, although a normal LDH level was associated with survival, it did not correlate with clinical response and also did not correlate with immune response. Collectively, these results reinforce the specificity of the result showing a significant association between an increased number of Melan-A–specific T cells and clinical outcome.

Expression of Melan-A in Resected Tumors After Immunization

It was conceivable that some patients developed PD despite immunization because of outgrowth of Melan-A–negative tumor cells. Posttreatment tumor samples were obtained from progressing tumors from three patients and analyzed by RT-PCR. Although the new metastasis that developed in patient 1 was negative for

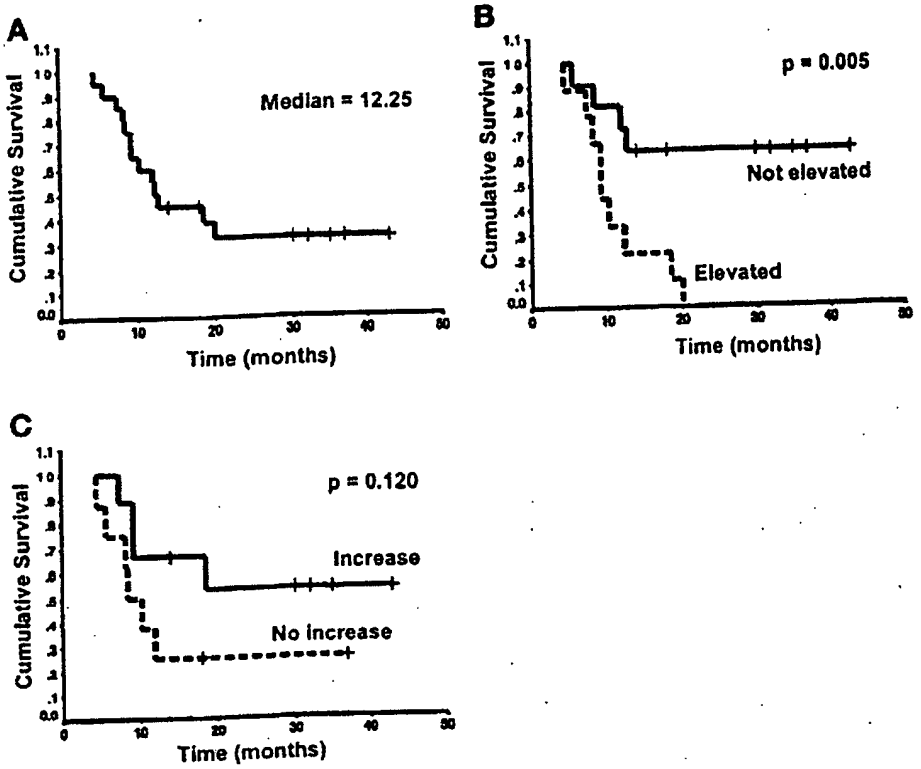

Fig 3. Overall survival for all patients (A), on the basis of serum lactate dehydrogenase greater than 200 U/L (B), and on the basis of increased Melan-A-specific interferon gamma-producing $CD8^+$ T cells (C) was determined using the Kaplan-Meier method. Differences between groups were compared using the log-rank test.

Melan-A expression, those samples from patients 4 and 6 retained detectable expression of Melan-A mRNA (Fig 1). These results indicate that, although outgrowth of antigen-negative tumors can occur, other mechanisms of resistance to immune destruction likely explain the lack of clinical response in other patients.

DISCUSSION

In this study we used Melan-A peptide-pulsed autologous PBMC + rhIL-12 as a vaccine to treat HLA-A2–positive patients with advanced melanoma. We observed a significant increase in Melan-A–specific IFN-γ–producing $CD8^+$ T cells after immunization, and found a statistical association between clinical response and the magnitude of the specific T-cell increase. Although it is difficult to compare across individual, small phase II studies, these results are similar to those that have been reported using antigen-loaded dendritic cells, but with a strategy that may be more straightforward to execute.

Preparation of the peptide-loaded PBMCs typically took 5 hours from phlebotomy to injection, and quality control of the cell product was facilitated by the lack of an extended in vitro culture period and absence of exposure to culture medium or serum proteins that is required for dendritic cell preparations. Conversely, dendritic cell vaccines have been prepared in batches and cryopreserved in individual doses in some studies, which obviates the need to prepare a fresh vaccine at each time point. Cryopreservation of vaccines has not yet been examined with our current approach. A comparative trial between PBMC/rhIL-12 and dendritic cell-based vaccination may, therefore, be of interest as the technologies continue to develop. Our results support the notion developed in preclinical models that IL-12 can contribute to effective antitumor immunity, and are consistent with the results of a recent adjuvant vaccine study using rhIL-12 in melanoma.[24]

We used a direct ex vivo ELISpot assay to assess antigen-specific T-cell responses in this study. Control experiments testing EBV reactivity from normal donors revealed that ELISpot analysis could be performed accurately on cryopreserved $CD8^+$ T cell samples immediately after thawing (H. Harlin and T. Gajewski, unpublished data). We found that background reactivity against the control HIV peptide varied among patients and to some extent among time points for an individual patient. The magnitude of increase in apparent Melan-A–reactive T cells would have been greater in some patients had the values obtained with the HIV control peptide not been subtracted. We believe that this experimental detail is critical because it normalizes the samples for background differences and provides an internal control for minor variation between individual vials of cryopreserved T cells. We also compared the Melan-A frequencies to those against an EBV control peptide, to determine whether the treatment was altering ELISpot results. We performed our analyses on purified $CD8^+$ T cells to control for variable numbers between patients and across time points. It is possible that we excluded subpopulations of $CD8^-$ T cells, $CD4^+$ T cells, and natural killer T cells that could have produced IFN-γ in response to Melan-A. Nonetheless, our results revealed a measurable and significant increase in Melan-A–specific T cells posttreatment. Our currently employed ELISpot assay is distinct from the assay used in our phase I trial of peptide-pulsed

Table 4. Statistical Correlates With Response or Survival

Parameter	Correlation With Response (P)	Correlation With Survival (P)
Positive DTH recall	.642	.130
Strong EBV pre-Rx*	.131	.491
Increased EBV post versus pre†	.290	.644
Strong Melan-A pre-Rx†	.644	.481
Increased Melan-A post versus pre†	.046	.120
Strong in vitro expansion of Melan-A§	.304	.565
LDH levels < 200	.99	.005

NOTE. Associations with response were determined using Fisher's exact test (two sided), except the differences between pre- and posttreatment, which were determined using an unpaired *t* test. Associations with survival were determined using the Kaplan-Meier method and log-rank test. Significant values are indicated in boldface.

Abbreviations: DTH, delayed-type hypersensitivity; EBV, Epstein-Barr virus; Rx, immunization; LDH, lactate dehydrogenase; HIV, human immunodeficiency virus; IL-2, interleukin-2.

*At least 90 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide.

†Changes between post- and prevaccination samples were calculated as the difference between the absolute number of specific spots and compared using an unpaired *t* test between clinical responders and nonresponders.

‡At least 40 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide.

§At least 90 spots per 10^5 $CD8^+$ T cells after subtraction of background against a control HIV peptide, after a 10-day in vitro expansion with Melan-A peptide-pulsed autologous $CD8^-$ cells and IL-2.

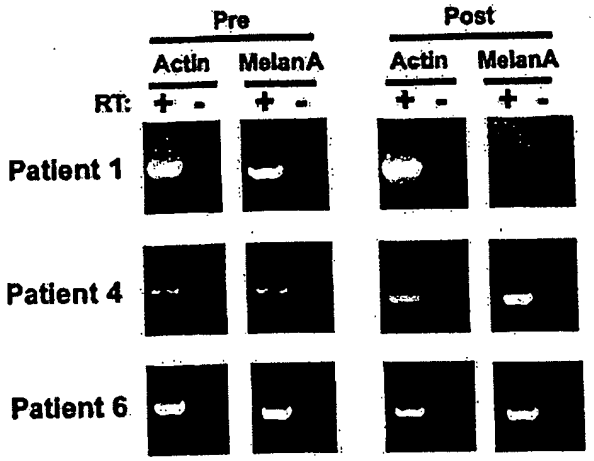

Fig 4. Melan-A expression in tumors that persisted after immunization. Three patients underwent surgical resection of lesions (after discontinuing the study), which were analyzed for Melan-A expression by qualitative reverse transcriptase polymerase chain reaction. Controls were analyzed without reverse transcriptase or with beta-actin primers.

PBMC + rhIL-12 and in other trials[21,25] in which in vitro expansion had been performed before assessment of IFN-γ production. Analysis of T-cell responses with minimal in vitro manipulation should most accurately reflect the status of those cells in vivo.

High frequencies of Melan-A–specific, IFN-γ–producing $CD8^+$ T cells were observed in some patients at study entry when they clearly had progressively growing melanoma. This observation indicates that the absolute frequency of functional T cells against a tumor antigen does not correlate with the behavior of the tumor. We also found no statistical association between this high frequency and clinical outcome; in fact, the two patients who experienced a CR had undetectable Melan-A–specific T cells before therapy. Although high frequencies of T cells reacting with a Melan-A tetramer have been detected in some normal donors,[26] those cells had a naïve surface phenotype and did not produce high levels of IFN-γ. What did correlate with clinical response in our current study is a meaningful increase in Melan-A–specific T cells posttreatment. These increases were modest (a net gain of 112 spots per 10^5 $CD8^+$ T cells on average), indicating either that a subtle alteration in the steady-state between the immune response and a growing tumor in favor of increased T-cell frequencies is sufficient to translate into tumor regression, or that another immune function that we are not measuring is contributing to the final event of tumor shrinkage. Tumor regressions without detectable increases in T-cell frequencies using standard assays have been observed in other studies.[27]

The median overall survival in our study was 12.25 months from treatment initiation, which is greater than the expected 6 to 9 months for this patient population. Although it was a relatively small study and subject to selection bias, most patients were pretreated and had visceral disease, one half of the patients had elevated serum LDH levels, and four patients had treated brain metastases. As has been seen in melanoma patients treated with standard therapies, we found that an elevated serum LDH level was a negative prognostic factor for survival. Whether this is reflective of tumor burden or the metabolic state of the tumor cells that have adapted to an anaerobic environment is unclear.

Some patients developed increases in Melan-A–specific T cells and developed progressive tumor growth despite retained expression of the antigen on posttreatment biopsies. This observation is similar to that seen in murine studies[28] and indicates mechanisms of tumor resistance downstream from initial T-cell priming, presumably within the tumor microenvironment. Potential explanations include poor T-cell trafficking to tumor sites, presence of negative regulatory cells, T-cell anergy or death, expression of inhibitory molecules by tumor cells, or downregulation of class I major histocompatibility complex or antigen-processing molecules.[29,30] Future studies should investigate definable mechanisms of tumor escape that allow tumor cells to resist elimination by antigen-specific T cells in vivo.

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REFERENCES

1. Boon T, Cerottini JC, Van den Eynde B, et al: Tumor antigens recognized by T lymphocytes. Annu Rev Immunol 12:337-365, 1994

2. Kawakami Y, Robbins PF, Wang RF, et al: Identification of tumor-regression antigens in melanoma. Important Adv Oncol 3-21, 1996

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3. Coulie PG, Brichard V, Van Pel A, et al: A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 180:35-42, 1994

4. Gajewski TF, Fallarino F: Rational development of tumor antigen-specific immunization in melanoma. Ther Immunol 2:211-225, 1995

5. Nestle FO, Alijagic S, Gilliet M, et al: Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4:328-332, 1998

6. Thurner B, Haendle I, Roder C, et al: Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 190:1669-1678, 1999

7. Banchereau J, Palucka AK, Dhodapkar M, et al: Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 61:6451-6458, 2001

8. Gajewski TF, Renauld JC, Van Pel A, et al: Costimulation with B7-1, IL-6, and IL-12 is sufficient for primary generation of murine antitumor cytolytic T lymphocytes in vitro. J Immunol 154:5637-5648, 1995

9. Mehrotra PT, Wu D, Crim JA, et al: Effects of IL-12 on the generation of cytotoxic activity in human CD8+ T lymphocytes. J Immunol 151:2444-2452, 1993

10. Trinchieri G: Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 13:251-276, 1995

11. Fallarino F, Uyttenhove C, Boon T, et al: Endogenous IL-12 is necessary for rejection of P815 tumor variants in vivo. J Immunol 156:1095-1100, 1996

12. Fallarino F, Gajewski TF: Cutting edge: Differentiation of antitumor CTL in vivo requires host expression of Stat1. J Immunol 163:4109-4113, 1999

13. Brunda MJ, Luistro L, Warrier RR, et al: Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178:1223-1230, 1993

14. Hallez S, Detremmerie O, Giannouli C, et al: Interleukin-12-secreting human papillomavirus type 16-transformed cells provide a potent cancer vaccine that generates E7-directed immunity. Int J Cancer 81:428-437, 1999

15. Fallarino F, Ashikari A, Boon T, et al: Antigen-specific regression of established tumors induced by active immunization with irradiated IL-12- but not B7-1-transfected tumor cells. Int Immunol 9:1259-1269, 1997

16. Vagliani M, Rodolfo M, Cavallo F, et al: Interleukin 12 potentiates the curative effect of a vaccine based on interleukin 2-transduced tumor cells. Cancer Res 56:467-470, 1996

17. Cavallo F, Signorelli P, Giovarelli M, et al: Antitumor efficacy of adenocarcinoma cells engineered to produce interleukin 12 (IL-12) or other cytokines compared with exogenous IL-12. J Natl Cancer Inst 89:1049-1058, 1997

18. Rao JB, Chamberlain RS, Bronte V, et al: IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: Enhancement by simultaneous B7-1 expression. J Immunol 156:3357-3365, 1996

19. Sumimoto H, Tani K, Nakazaki Y, et al: Superiority of interleukin-12-transduced murine lung cancer cells to GM- CSF or B7-1 (CD80) transfectants for therapeutic antitumor immunity in syngeneic immunocompetent mice. Cancer Gene Ther 5:29-37, 1998

20. Fallarino F, Uyttenhove C, Boon T, et al: Improved efficacy of dendritic cell vaccines and successful immunization with tumor antigen peptide-pulsed peripheral blood mononuclear cells by coadministration of recombinant murine interleukin-12. Int J Cancer 80:324-333, 1999

21. Gajewski TF, Fallarino F, Ashikari A, et al: Immunization of HLA-A2+ melanoma patients with MAGE-3 or MelanA peptide-pulsed autologous peripheral blood mononuclear cells plus recombinant human interleukin 12. Clin Cancer Res 7:895s-901s, 2001

22. Balch CM, Buzaid AC, Atkins MB, et al: A new American Joint Committee on Cancer staging system for cutaneous melanoma. Cancer 88:1484-1491, 2000

23. Eton O, Legha SS, Moon TE, et al: Prognostic factors for survival of patients treated systemically for disseminated melanoma. J Clin Oncol 16:1103-1111, 1998

24. Lee P, Wang F, Kuniyoshi J, et al: Effects of interleukin-12 on the immune response to a multipeptide vaccine for resected metastatic melanoma. J Clin Oncol 19:3836-3847, 2001

25. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al: Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 4:321-327, 1998

26. Pittet MJ, Valmori D, Dunbar PR, et al: High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. J Exp Med 190:705-715, 1999

27. Marchand M, van Baren N, Weynants P, et al: Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int J Cancer 80:219-230, 1999

28. Wick M, Dubey P, Koeppen H, et al: Antigenic cancer cells grow progressively in immune hosts without evidence for T cell exhaustion or systemic anergy. J Exp Med 186:229-238, 1997

29. Ferrone S, Marincola FM: Loss of HLA class I antigens by melanoma cells: Molecular mechanisms, functional significance and clinical relevance. Immunol Today 16:487-494, 1995

30. Marincola FM, Jaffee EM, Hicklin DJ, et al: Escape of human solid tumors from T-cell recognition: Molecular mechanisms and functional significance. Adv Immunol 74:181-273, 2000